

Phylogeography of *Francisella tularensis* subsp. *holarctica*, Europe

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Francisella tularensis subsp. *holarctica* isolates from Austria, Germany, Hungary, Italy, and Romania were placed into an existing phylogeographic framework. Isolates from Italy were assigned to phylogenetic group B.FTNF002-00; the other isolates, to group B.13. Most *F. tularensis* subsp. *holarctica* isolates from Europe belong to these 2 geographically segregated groups.

Francisella tularensis is the etiologic agent of tularemia and a highly virulent category A biothreat agent (1,2). The most widely distributed subspecies is *F. tularensis* subsp. *holarctica*, which is found throughout much of the Northern Hemisphere and is the only subspecies found in Europe (3). Despite its wide geographic distribution, *F. tularensis* subsp. *holarctica* contains low genetic diversity, which indicates recent emergence (4). A recent global phylogeographic analysis (5), and several subsequent analyses (6–9), assigned most isolates from Europe to 2 phylogenetic groups: B.FTNF002-00 and B.13 (includes multiple subclades descended from branch B.13 [5,6,8]; branch and subclade nomenclature from [5] has been shortened by removing Br and extra 0s from individual branch and subclade names). These groups appear to be geographically segregated: only isolates from B.FTNF002-00 have been reported from the western European countries of Spain, France, and Switzerland,

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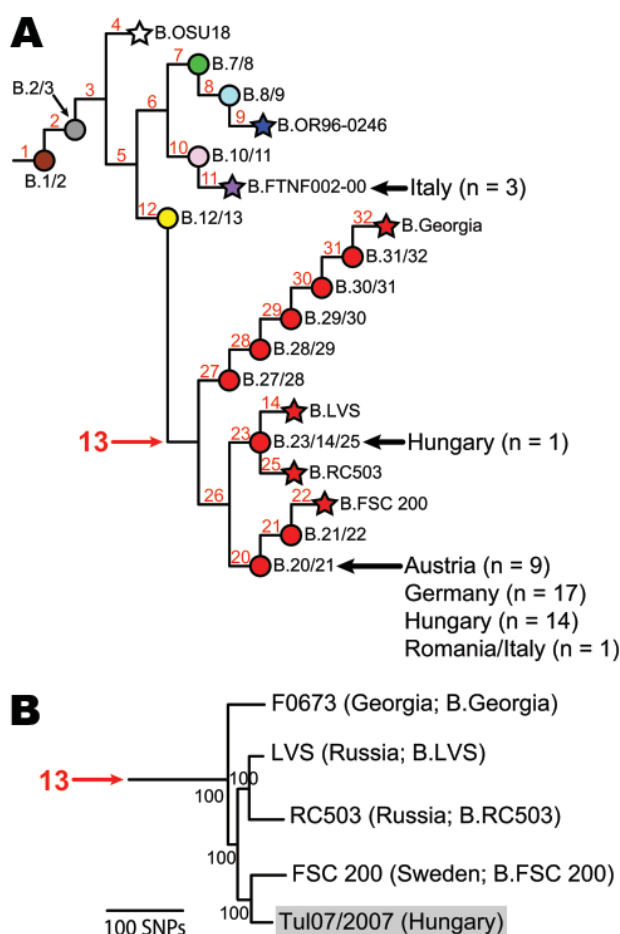


Figure 1. Existing phylogeny of *Francisella tularensis* subsp. *holarctica*. A) Single nucleotide polymorphism (SNP)-based phylogeny of *F. tularensis* subsp. *holarctica* derived from previous studies (5,6,8). Terminal subgroups representing sequenced strains are shown as stars, and intervening nodes representing collapsed branches are indicated by circles. Subclades within group B.13 are depicted in red. Isolates from Austria, Germany, Hungary, Italy, and Romania (n = 45) were assigned to existing subclades (black arrows) by using existing canonical SNP assays (5,8). B) Maximum parsimony phylogeny constructed by using SNPs discovered from 6 *F. tularensis* whole-genome sequences, including 5 strains from group B.13 and an outgroup strain, OSU18 (not shown). This phylogeny was rooted by using OSU18, and bootstrap values were based on 1,000 simulations by using a heuristic search. The newly sequenced Hungarian strain (Tul07/2007) is highlighted in gray.

whereas B.13 is the only or dominant type reported from the Czech Republic, Finland, Georgia, Russia, Slovakia, and Ukraine (5–9). We provide additional information about the geographic distribution of these 2 groups using existing phylogenetic signatures (5,8) to place 45 isolates from Austria, Germany, Hungary, Italy, and Romania (online Appendix Table, wwwnc.cdc.gov/EID/article/18/2/11-

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1305-TA1.htm) into the existing global phylogeographic framework.

The Study

All of the isolates were assigned to group B.FTNF002–00 or to group B.13. All 3 isolates from Italy were assigned to group B.FTNF002–00 (Figure 1, panel A). Although the sample size was small, these isolates were obtained in 3 different years (online Appendix Table), which suggests that this group is ecologically established in Italy. These results increase the known geographic distribution of this group, which appears to be the dominant clone in western Europe (Figure 2, panel A, purple shading). All 42 isolates from Austria, Germany, Hungary, and Romania were assigned to group B.13 (Figure 1, panel A), further demonstrating that B.13 is the most prevalent group of *F. tularensis* subsp. *holarctica* in central and eastern Europe (Figure 2, panel A, red shading). Within group B.13, one isolate from Hungary was assigned to subclade B.23/14/25 (Figure 1, panel A); isolates from Finland, Russia, and Sweden were previously assigned to this subclade (6,8)

(Figure 2, panel B). However, the other 41 isolates were assigned to subclade B.20/21 (Figure 1, panel A).

We identified new genomic signatures to provide increased genetic resolution within subclade B.20/21. Next-generation sequencing technology (Illumina Inc., San Diego, CA, USA) was used to sequence the genome of an isolate from Hungary (Tul07/2007, GenBank accession no. SRX025133) assigned to subclade B.20/21. Putative single nucleotide polymorphisms (SNPs) were identified in the resulting sequence and the genomes of 4 other strains previously assigned to group B.13 (LVS, AM233362.1; FSC 200, AASP00000000; RC503, SRX000104; Georgia F0673, SRX025885) by using an existing bioinformatics pipeline (5). The more distantly related strain OSU18 (CP000437.1) genome was also included as an outgroup. A maximum-parsimony tree was constructed by using the resulting ≈ 700 putative SNPs and PAUP 4.0b10 software (Sinauer Associates, Inc., Sunderland, MA, USA) (Figure 1, panel B). Most of the putative SNPs separated OSU18 from the B.13 strains (data not shown), but the remaining putative SNPs provided resolution among the B.13 strains,

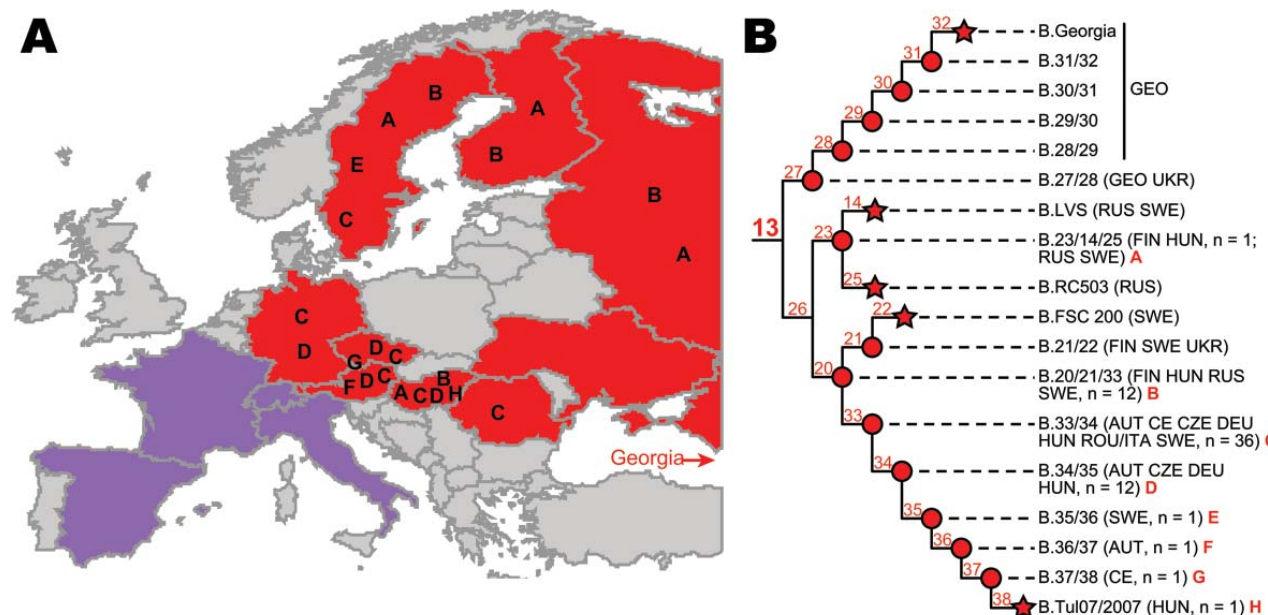


Figure 2. Detailed geographic distribution and phylogeny of *Francisella tularensis* subsp. *holarctica* subclades within group B.13. A) Countries from which groups B.13 and B.FTNF002–00 have been reported. Countries of origin for isolates assigned to select subclades within group B.13 are indicated by the letters A–H. Red and purple shading indicates the known geographic distributions of groups B.13 and B.FTNF002–00, respectively, in this and previous studies (5–9). The country of Georgia, which also contains isolates from group B.13 but is not depicted in the map, is indicated by red text and a red arrow pointing toward its location. Isolates assigned to other phylogenetic groups within *F. tularensis* subsp. *holarctica* have been reported from some of these countries (5,8), but most isolates from these countries are from groups B.13 and B.FTNF002–00. B) Single nucleotide polymorphism–based phylogeny of previously (5,6,8) and newly identified subclades within the B.13 group of *F. tularensis* subsp. *holarctica*. Terminal subgroups representing sequenced strains are shown as stars, and intervening nodes representing collapsed branches are indicated by circles. The countries of origin for isolates assigned to each subclade are indicated: AUT, Austria; CE, central Europe, unknown country; CZE, Czech Republic; DEU, Germany; FIN, Finland; GEO, Georgia; HUN, Hungary; ITA, Italy; ROU, Romania; RUS, Russia; SWE, Sweden; UKR, Ukraine). For mapping purposes, letters are assigned to a previously identified subclade that contains a new isolate from Hungary now assigned to that subclade (A) and newly identified subclades (B–H). The number of isolates listed for each subclade refers only to isolates examined directly in this study (online Appendix Table, wwwnc.cdc.gov/EID/article/18/02/11-1305-TA1.htm).

including 20 putative SNPs specific to the branch leading to the strain from Hungary (Figure 1, panel B). Consistent with previous analyses (Figure 1, panel A), the strain from Hungary clustered as a sister taxon to strain FSC 200 (Figure 1, panel B).

To show additional phylogenetic structure within subclade B.20/21, we designed genotyping assays targeting the 20 putative SNPs along the branch leading to the strain from Hungary (Figure 1, panel B) and screened them across 64 isolates assigned to subclade B.20/21. This analysis included the 41 isolates from Austria, Germany, Hungary, and Romania, as well as 23 additional isolates from central Europe, the Czech Republic, Finland, Russia, and Sweden that were previously assigned to this subclade (6,8) (online Appendix Table). The assays were constructed and performed as described (5) by using an annealing temperature of 60°C. All 20 SNPs were laboratory confirmed, and 52 of the isolates were assigned to 6 new subclades (B.33/34, B.34/35, B.35/36, B.36/37, B.37/38, and B.Tul07/2007); the 12 other isolates remained in the basal subclade, now identified as B.20/21/33 (Figure 2, panel B; online Appendix Table). Information about assays targeting canonical SNPs for the branches leading to the 6 new subclades are presented in the Table.

Conclusions

Our results are consistent with complex dispersal patterns within the B.13 group of *F. tularensis* subsp. *holarctica*. Several of the B.13 subclades identified in this study are broadly distributed throughout central and eastern Europe (Figure 2, panel A), including subclades

B.20/21/33, B.33/34, and B.34/35. All of the new subclades containing >1 isolate have representatives from multiple countries (Figure 2, panel B). Other previously identified B.13 subclades, including B.27/28, B.LVS, B.23/14/25, and B.21/22 are also broadly distributed (Figure 2, panel A).

This study and previous studies have increased understanding of *F. tularensis* subsp. *holarctica* in Europe by placing isolates from multiple countries into the existing global phylogeographic framework. As a result, the genetic background is becoming defined for each country (i.e., the specific subtypes reported from each country). This information can be useful for identifying intentional (e.g., bioterrorism) or unintentional movement of *F. tularensis* subsp. *holarctica* between countries. For example, the isolate from Romania examined in this study was actually isolated in Italy from an infected hare that was shipped from Romania for hunting. Genotyping results are consistent with a Romanian origin for this isolate because it was assigned to the B.13 group that is widespread in central and eastern Europe (Figure 2, panel A) and not to the B.FTNF002–00 group, to which the isolates from Italy were assigned (Figure 1, panel A).

Understanding global phylogeographic patterns is possible only if isolates from multiple geographic locations are placed within the same framework (i.e., examined with the same genomic signatures). Because *F. tularensis* is genetically monomorphic and highly clonal, SNPs are preferred signatures for determining phylogenetic structure within this species (3). Vogler et al. (5) conducted the first SNP-based global phylogeographic analysis of *F. tularensis*. Subsequent studies (6–8) have used the SNP

Table. Melt-MAMA primers targeting canonical SNPs for 6 new phylogenetic branches in a study of *Francisella tularensis* subsp. *holarctica*, Europe*

SNP	SCHU† S4 position	SNP state, D/A‡	Primers, 5' → 3'§	Con, µM¶	T _m , °C
B.33	78,382	T/C	A: ATTGCTACTTCTATTTACGCCAACAG	0.20	74.3
			D: GGGGCGGGGCGGGGCATTGCTACTTCTATTTACGCCAAGAA	0.20	79.0
			C: TGTGAACAACCAAGTTGGCTT	0.20	
B.34	766,614	A/G	A: GTAGCGAGCATTATTTGCTGGTTC	0.40	69.2
			D: GGGGCGGGGCGGGGCTAGCGAGCATTATTTGCTGGGTT	0.20	78.6
			C: ATAAACTATAAATTTACATAAAATGAAAATTCTC	0.20	
B.35	239,479	A/C	A: GCCTTAATCTAGTATTTTCGCTTATCTCC	0.40	70.3
			D: GGGGCGGGGCGGGGCGCCTTAATCTAGTATTTTCGCTTATCACA	0.20	75.5
			C: CGGGCTCTAAAATAAGATTTAAGTTAGTAAGT	0.20	
B.36	1,599,292	A/C	A: TATTATAGTTTCTAAAACAGTCTAATTAATTTTG	0.60	69.0
			D: GGGGCGGGGCGGGGCTATTATAGTTTCTAAAACAGTCTAATTAATTGTT	0.20	73.9
			C: GTTCGACCATGACTACAGTGTG	0.20	
B.37	318,602	T/C	A: AACATTTTAGGAACTCTACGATGATAAACTTAAC	0.20	69.7
			D: GGGGCGGGGCGGGGCCATTTAGGAACTCTACGATGATAAACTTGAT	0.20	75.9
			C: GAAATATCTCAATGAAATCTAATTTAACTAAAATCAC	0.20	
B.38	166,885	C/T	A: ATGCCATCAGCCATTTACTACTACA	0.20	73.7
			D: GGGGCGGGGCGGGGCCATTCAGCCATTTACTACTCCCG	0.20	80.1
			C: CTTCCCTGATTTTCTAAGTTCTGCTTG	0.20	

*Melt-MAMA, melt-mismatch amplification mutation assay; SNP, single nucleotide polymorphism; con, concentration; T_m, melting temperature for ancestral and derived Melt-MAMA amplification products.

†SCHU strain GenBank accession no. NC_006570.

‡SNP states are presented according to their orientation in the SCHU S4 reference genome (AJ749949.2); D, derived SNP state; A, ancestral SNP state.

§D, derived allele primer; A, ancestral allele primer; C, common primer; primer tails and antepenultimate mismatch bases are in lower case.

¶Final concentration of each primer in Melt-MAMA genotyping assays.

signatures described by Vogler et al. (5) and new SNPs discovered from new whole-genome sequences or multiple sequence typing data to further refine phylogeographic patterns within *F. tularensis*, particularly *F. tularensis* subsp. *holarctica*. These new signatures, when screened across diverse isolate collections, have identified new subclades within preexisting subclades. This pattern will continue as whole-genome sequencing becomes less expensive and more widely available. As a result, the nomenclature of phylogenetic groups within *F. tularensis* and the particular subclade to which a given isolate is assigned are constantly changing and will continue to change, which makes comparison of results and findings across different studies difficult. To address this problem, we have included all known *F. tularensis* subsp. *holarctica* SNP-based phylogenetic groups within our phylogenetic trees (Figure 1, panel A; Figure 2, panel B), including those discovered by other researchers. In addition, for the isolates analyzed in this study (online Appendix Table), where applicable, we have listed the phylogenetic groups to which they were assigned in previous studies.

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